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FULL-TEXT ARTICLE**

RNA aptamers that specifically bind to the Ras-binding domain of Raf-1.

Kimoto M, Sakamoto K, Shirouzu M, Hirao I, Yokoyama S.

Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Japan.

RNA aptamers that bind to the Ras-binding domain (RBD) of a proto-oncogene product, Raf-1, were isolated from a pool of random sequences using a glutathione S-transferase-fused RBD (GST-RBD). The RNA molecules bind to the GST-RBD, but not to GST, with dissociation constants of about 300 nM. In contrast, these RNA aptamers do not bind to the Ras-binding domain of the RGL protein, which is also known to be activated by Ras. The aptamers actually compete with Ras for binding to the Raf-1 RBD. The anti-Raf-1 aptamers may be used to specifically inhibit the Ras-Raf interaction in the complicated signaling network in mammalian cells.

PMID: 9883908 [PubMed - indexed for MEDLINE]

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09/654, 28/



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☐ 1: J Mol Evol 1994 Dec;39(6):655-60

Related Articles, Links

Amino acid sequence of the Homo sapiens brain 21-23-kDa protein (neuropolypeptide h3), comparison with its counterparts from Rattus norvegicus and Bos taurus species, and expression of its mRNA in different tissues.

Seddiqi N, Bollengier F, Alliel PM, Perin JP, Bonnet F, Bucquoy S, Jolles P, Schoentgen F.

Laboratoire des proteines, CNRS URA 1188, Universite Paris 5, France.

The amino acid sequence of neuropolypeptide h3 from Homo sapiens brain has been determined. It revealed that h3 is the exact counterpart of the 21-kDa protein from Bos taurus brain and the 23-kDa protein from Rattus norvegicus brain: The three proteins belong to the same 21-23-kDa protein family. Multiple tissue Northern blots showed that the mRNA encoding the 21-23-kDa protein is expressed in different amounts according to tissues and species; it is particularly abundant in Rattus norvegicus testis.

MeSH Terms:

- Amino Acid Sequence
- Animal
- Base Sequence
- Cattle
- Comparative Study
- Human
- Molecular Sequence Data
- Nerve Tissue Proteins/genetics*
- Proteins/genetics*
- Rats
- Sequence Alignment
- Support, Non-U.S. Gov't

Substances:

- protein H(3)
- Proteins
- Nerve Tissue Proteins

Secondary source id:

- GENBANK/S76773

PMID: 7807553 [PubMed - indexed for MEDLINE]

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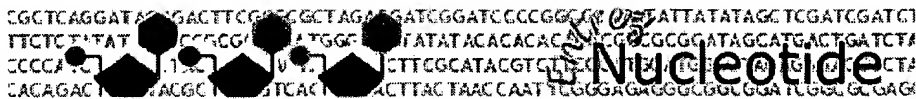
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Search for

☐ 1: X75252. H.sapiens phospho...[gi:406289]

[Links](#)

LOCUS HSPEABP 1444 bp mRNA linear PRI 25-AUG-1995
 DEFINITION H.sapiens phosphatidylethanolamine binding protein mRNA.
 ACCESSION X75252
 VERSION X75252.1 GI:406289
 KEYWORDS phosphatidylethanolamine-binding protein.
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 Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
 REFERENCE 1 (bases 1 to 1444)
 AUTHORS Tohdoh,N., Tojo,S., Agui,H. and Ojika,K.
 TITLE Sequence homology of rat and human HCNP precursor proteins, bovine
 phosphatidylethanolamine-binding protein and rat 23-kDa protein
 associated with the opioid-binding protein
 JOURNAL Brain Res. Mol. Brain Res. 30 (2), 381-384 (1995)
 MEDLINE 95364631
 PUBMED 7637590
 REFERENCE 2 (bases 1 to 1444)
 AUTHORS Tohdoh,N.
 TITLE Direct Submission
 JOURNAL Submitted (08-SEP-1993) N. Tohdoh, Sumitomo Pharmaceuticals Co.,
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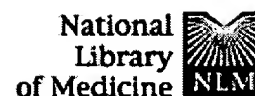
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☐ 1: Brain Res Mol Brain Res 1995 Jun;30(2):381-4

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ELSEVIER SCIENCE
FULL-TEXT ARTICLE

Sequence homology of rat and human HCNP precursor proteins, bovine phosphatidylethanolamine-binding protein and rat 23-kDa protein associated with the opioid-binding protein.

Tohdoh N, Tojo S, Agui H, Ojika K.

Discovery Research Laboratories III, Sumitomo Pharmaceuticals Research Center, Osaka, Japan.

The hippocampal cholinergic neurostimulating peptide (HCNP) enhances acetylcholine synthesis in rat medial septal tissues. We have cloned the cDNAs of the precursor proteins of rat and human HCNP and deduced their respective amino acid sequences. The HCNP sequences aligned at the N-terminal regions of their precursors. The deduced amino acid sequences showed homology with those of the bovine brain phosphatidylethanolamine-binding protein and the rat protein associated with the opioid-binding protein. These observations suggest that the HCNP precursor proteins may have multiple functions.

MeSH Terms:

- Acetylcholine/biosynthesis
- Amino Acid Sequence
- Animal
- Base Sequence
- Carrier Proteins/genetics*
- Cattle
- Cholinergic Agents/metabolism*
- DNA, Complementary
- Human
- Molecular Sequence Data
- Neuropeptides/metabolism*
- Neuropeptides/genetics
- Phosphatidylethanolamines/metabolism*
- Protein Precursors/metabolism*
- Rats
- Receptors, Opioid/metabolism*

Substances:

- Acetylcholine
- hippocampal cholinergic neurostimulating peptide
- Receptors, Opioid
- Protein Precursors
- Phosphatidylethanolamines
- Neuropeptides
- DNA, Complementary
- Cholinergic Agents
- Carrier Proteins

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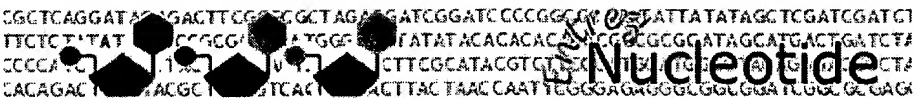
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- GENBANK/X75253
- GENBANK/X75252

PMID: 7637590 [PubMed - indexed for MEDLINE]

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Search for

☐ 1: X85033. H.sapiens mRNA fo...[gi:704464]

[Links](#)

LOCUS HSRNAPBP 570 bp mRNA linear PRI 07-FEB-1997
 DEFINITION H.sapiens mRNA for phosphatidylethanolamine binding protein.
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 SOURCE Homo sapiens.
 ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
 Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 570)
 AUTHORS Moore,C., Perry,A.C., Love,S. and Hall,L.
 TITLE Sequence analysis and immunolocalisation of
 phosphatidylethanolamine binding protein (PBP) in human brain
 tissue

JOURNAL Brain Res. Mol. Brain Res. 37 (1-2), 74-78 (1996)

MEDLINE 96346615

PUBMED 8738137

REFERENCE 2 (bases 1 to 570)

AUTHORS Hall,L.

TITLE Direct Submission

JOURNAL Submitted (28-FEB-1995) L. Hall, Dept. of Biochemistry, Univ. of
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FEATURES Location/Qualifiers

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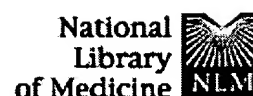
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Sequence analysis and immunolocalisation of phosphatidylethanolamine binding protein (PBP) in human brain tissue.

Moore C, Perry AC, Love S, Hall L.

Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, UK.

We have cloned and sequenced cDNA corresponding to human brain phosphatidylethanolamine binding protein (PBP) and have used the resultant data to generate PBP-specific anti-peptide antisera for immunohistochemical studies. The distribution of PBP was assessed immunohistochemically in sections from multiple regions of neonatal brain and spinal cord, including spinal nerve roots. Strong PBP immunoreactivity was present in the cytoplasm of oligodendrocytes in the white matter and Schwann cells in the nerve roots. There was only weak immunostaining within the neurones and neuropil. The findings are in keeping with a role for PBP in the organisation of phospholipids in the myelin sheath.

MeSH Terms:

- Base Sequence
- Brain/metabolism*
- Carrier Proteins/metabolism*
- Carrier Proteins/immunology
- Cerebral Cortex/metabolism
- Human
- Immunohistochemistry
- Molecular Sequence Data
- Myelin Sheath/metabolism*
- Phosphatidylethanolamines/metabolism*
- Support, Non-U.S. Gov't

Substances:

- Phosphatidylethanolamines
- Carrier Proteins

Secondary source id:

- GENBANK/X85033

PMID: 8738137 [PubMed - indexed for MEDLINE]

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PubMed Nucleotide Protein Genome Structure PopSet Taxonomy OMIM Bio

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☐ 1: U43206. Mus musculus phos...[gi:1517863]

Links

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DEFINITION Mus musculus phosphatidylethanolamine binding protein mRNA,
complete cds.
ACCESSION U43206
VERSION U43206.1 GI:1517863
KEYWORDS .
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ORGANISM Mus musculus
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE 1 (bases 1 to 1180)
AUTHORS Lin,B. and Frischauf,A.M.
TITLE Cloning of mouse phosphatidylethanolamine binding protein gene
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1180)
AUTHORS Lin,B. and Frischauf,A.M.
TITLE Direct Submission
JOURNAL Submitted (13-DEC-1995) Biaoyang Lin, MAMM, ICRF, 44 Lincoln's Inn
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DH345.77

From: Yu, Misook
Sent: Wednesday, October 02, 2002 3:32 PM
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Subject: requesting journal article for 09/654,281

TRENDS IN BIOCHEMICAL SCIENCES, (1994 Nov) 19 (11) 474-80

Examiner Misook Yu, Ph.D.
703-308-2454 (Phone)
Art Unit 1642
CM1-8E18 (Room)
CM1-8E12 (Mail Box)

Not relevant '0-3-02 my

RAF PROTEIN serine/threonine (Ser/Thr) kinases are essential for growth and development in worms, flies, frogs and mammals. Receptors that regulate Raf kinases include members of the serpentine family that contain seven transmembrane helices (STMRs), transmembrane tyrosine kinase receptors (RTKs), and cytokine receptors that regulate intracellular protein tyrosine kinases (PTKs). Typically, these receptors stimulate Raf kinases indirectly by a process that involves the small GTP-binding protein Ras. Like Ras, Raf kinases were first discovered as gain-of-function mutants with the ability to transform cells oncogenically¹.

Comparison of mutant *raf* with normal *raf* showed that the 5'-half of the gene was generally deleted in the mutant and that the minimal transforming sequence corresponded to the kinase domain². As normal Raf from quiescent cells was inactive as a kinase and the oncogenic forms had constitutive activity, the missing part encoding the amino-terminal half of the molecule was likely to function in autoinhibition. In fact, expression of the amino-terminal half of Raf in the absence of a functional kinase domain antagonized growth factor- or oncogene-induced Raf activation, illustrating that mutations of *raf* can also generate inhibitors of signal transduction. The transforming activity of gain-of-function mutants of *raf*, as well as their ability to abrogate growth factor dependence of established cell lines, led to the prediction of a function for c-Raf-1 as a cytoplasmic signal transducer of growth factor receptors many years ago¹. It is the work of the past few years, however, that has established the role of Raf as a central player in signal transduction.

The Raf protein kinase family

The Raf kinases are evolutionarily highly conserved, a fact that facilitated the isolation of homologues of Raf in invertebrates such as *Drosophila* (D-Raf)³ and *Caenorhabditis elegans* (Ce-Raf)⁴. Genetic experiments in these organisms have revealed the existence of one functional gene, whereas three such genes were described in vertebrates, *A-raf*, *B-raf* and *c-raf-1* (Fig. 1). Alternative splicing may contribute to the diversity of the Raf kinase family, as has been reported for *c-raf-1* in chicken⁵. All Raf proteins

G. Daum, I. Eisenmann-Tappe, H.W. Fries, J. Troppmair and U. R. Rapp are at the Institute of Medical Radiobiology and Cell Biology, University of Würzburg, Versbacher Strasse 5, D-97078 Würzburg, Germany.

The ins and outs of Raf kinases

Günter Daum, Iris Eisenmann-Tappe,
Hans-Werner Fries, Jakob Troppmair
and Ulf R. Rapp

Raf kinases are signal-integrating enzymes that have the ability to switch tyrosine kinase signalling to serine/threonine phosphorylation and connect growth factor receptors with transcription factors. The connection involves a cascade of protein kinases that is essential for cellular proliferation and differentiation of species ranging from worms to humans. This cascade also mediates transformation by most oncogenes.

share three highly conserved regions (CR1–3) embedded in variable sequences. The variable sequences are similarly conserved between species, suggesting that they have isozyme-specific functions. CR1 has a composite structure consisting of a binding domain for Ras followed by a zinc-finger motif of the type Cx₂Cx₃Cx₂C. Computer modelling of this sequence suggests an amphipathic α -helix tethered by Zn²⁺-coordination of the four sulphhydryl groups. Since Raf kinases are localized in the cytosol of unstimulated cells, it is reasonable to expect that the hydrophobic part of the zinc finger is buried within the molecule. CR2 is rich in serine and threonine residues, some of which are regulatory phosphorylation sites. The catalytic domain of the Raf kinases resides in CR3, which shows closer relatedness to *src* family PTKs than to other protein kinases⁶. Mutant analysis of *c-raf-1* in NIH3T3 cells supports the proposed function of CR1 and CR2 as the regulatory part of the Raf molecule^{1,2}. Amino-terminal extension, alteration of the zinc-finger structure, mutation of Ser259 in CR2 or disruption of this region by linker insertion, and finally deletion of the entire amino-terminal half all resulted in oncogenic conversion of the *c-raf-1* gene, which correlated with constitutive kinase activity. The *trans*-repression function of CR1 is not due to CR1–CR3 interaction but to competition of CR1 with endogenous Raf for Ras binding^{7–9}.

Receptor coupling of the cytoplasmic kinase cascade: the role of Ras

The only known substrate for Raf is another protein kinase, MAP/ERK

kinase (MEK), which it stimulates to activate yet another protein kinase, mitogen-activated protein kinase (MAPK, also known as extracellular-signal-regulated kinase, ERK)⁹. These three enzymes form what is now known as the cytoplasmic kinase cascade, which represents an essential pathway of mitogenic signal transduction in many cell types^{10,11}. Receptors gain access to this cascade through a variety of mechanisms. The best studied, and for many receptors the essential connector, is Ras. Ras is regulated by multiple components to form the Ras cycle, which occurs between an inactive, GDP-bound and an active, GTP-bound form of Ras mediated by GDP–GTP exchange factors (GEFs) and regulatory proteins that stimulate the intrinsic GTPase activity of Ras (GTPase activating proteins, GAPs)¹². The Ras cycle is connected to activated receptors by adaptor proteins, such as Grb2, which recruit the GEF Sos, resulting in increased steady-state levels of Ras–GTP¹³.

Only the GTP-bound form of Ras can bind effector molecules, such as Raf, GAP and phosphoinositide (PI) 3-kinase^{14,15}. Comparison of Ras effector binding and Ras transforming activity showed the best correlation with Raf kinase, indicating that Raf kinase represents the critical transformation effector of Ras⁹. Raf was essential for Ap-1/ets-mediated transcription induced by Ras, growth factors or protein kinase C (PKC) in transient-reporter-gene assays. Expression of CR1 inhibited transcription of the reporter gene, indicating that it contains the site required for Ras-dependent regulation. These experiments further suggested that the

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zinc-finger structure was important for Ras-Raf interaction, since a mutation of Cys168, the most carboxy-terminal cysteine in the structure, abolished the dominant-negative effect of CR1 (Ref. 8). Consistent with these findings was the observation that the part of Ras that effectors bind to also binds to wild-type CR1, but not to mutant CR1 *in vivo*⁹. Examination of Ras-Raf binding in the yeast two-hybrid system has revealed an 81 amino acid sequence (residues 51–131 of c-Raf-1) as the Ras-binding domain (RBD)⁹. This sequence is highly conserved between the three mammalian Raf isozymes, and was also found in D-Raf. Interestingly, a point mutation in the CR1 region of D-Raf (Arg217Leu) was already known to interfere with the *sevenless* RTK signalling pathway, resulting in obstruction of eye development in *Drosophila*³. The homologous mutation in c-Raf-1 (Arg89Leu) was sufficient to abolish Ras-binding activity of the RBD¹⁶. The Arg89Leu mutation in the RBD did not increase kinase activity, whereas the Cys168 mutation in the zinc-finger structure activated c-Raf-1 to an intermediate level, presumably by causing partial unfolding of inhibitory regions (J. T. Bruder, unpublished). The surprising finding that the Cys168 mutation, although not localized in the RBD, also abolished Raf-Ras interaction indicates the need for conformational integrity of the RBD-surrounding regions for Ras binding *in vivo*.

Attempts to activate purified Raf with Ras-GTP failed, indicating a need for additional factors. Earlier experiments had also indicated that Ras was insufficient to activate Raf kinase fully. For example, Reed *et al.* showed that, in contrast to the situation in Src-transformed cells, activation of Raf in Ras-transformed fibroblasts was still growth factor dependent⁷. Similarly, coexpression of *c-raf-1* with oncogenic *ras* in Sf9 insect cells using recombinant baculovirus failed to activate Raf completely, which was achieved following additional coexpression of *v-src*⁷. It was suggested that the additional factor may be a Raf kinase kinase, present in activated RTK-PTK complexes.

Protein kinase C- α (PKC- α) is a likely candidate for such an additional factor, since it can phosphorylate and activate c-Raf-1 (Ref. 17). In addition, both kinases translocate to the membrane upon activation. We also have to consider the contribution of a lipid cofactor, as the Raf zinc finger is related to a similar structure in PKC that is known to form part of the phorbol-ester/diacylglycerol (DAG)-binding site in PKC. However, DAG and phorbol esters did not bind to c-Raf-1 *in vitro*¹⁸.

Taken together, these findings led to the proposal that Ras transports Raf, perhaps aided by a cofactor, to its activator kinase(s) in the membrane^{7,19}. Recent experiments have directly tested the idea that membrane localization of Raf would result in kinase activation. A fusion protein was constructed consisting of c-Raf-1 plus the 17 carboxy-terminal residues of Ras, which comprise its membrane-localization domain. This membrane-located Raf fusion protein was activated to an intermediate level and was resistant to inhibition by dominant-negative Ras, but

full activation still required stimulation by growth factors¹⁵.

Is phosphorylation of Raf required for its activation?

Any physiological activation of c-Raf-1 is paralleled by its hyperphosphorylation, which is easily detected by a retardation of the protein in sodium dodecyl sulphate polyacrylamide gel electrophoresis. Phosphorylation of c-Raf-1 occurs mainly on serine residues when serum-starved cells are stimulated by insulin or growth factors such as platelet-derived growth factor (PDGF). Only traces of phosphothreonine or phosphotyrosine were found under these conditions²⁰. Phosphopeptide maps of immunoprecipitated c-Raf-1 from cells stimulated with a variety of factors were almost identical, suggesting that signalling induced the same c-Raf-1 kinase kinase(s)¹⁹. Morrison *et al.*²¹ identified three major phosphorylation sites of c-Raf-1 isolated from PDGF-stimulated NIH3T3 cells; these were the serine residues at positions 43, 259 and 621 (Fig. 1). Ser621 is also

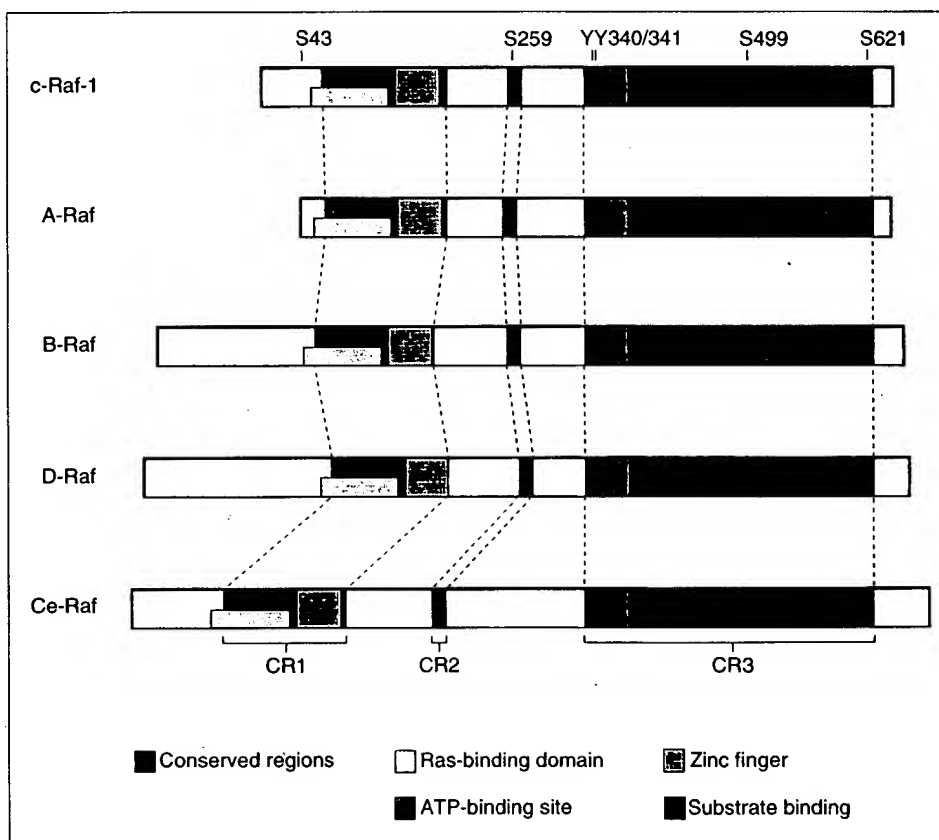


Figure 1

Structure of Raf kinases: c-Raf-1, A-Raf and B-Raf are mammalian isozymes; D-Raf and Ce-Raf are from *Drosophila* and *C. elegans*, respectively. CR1 (residues 62–196; numbers refer to c-Raf-1), CR2 (residues 255–268) and CR3 (residues 331–625) represent conserved regions. Note that phosphorylation sites given for c-Raf-1 are not necessarily present on the other Raf kinases. See text for details.

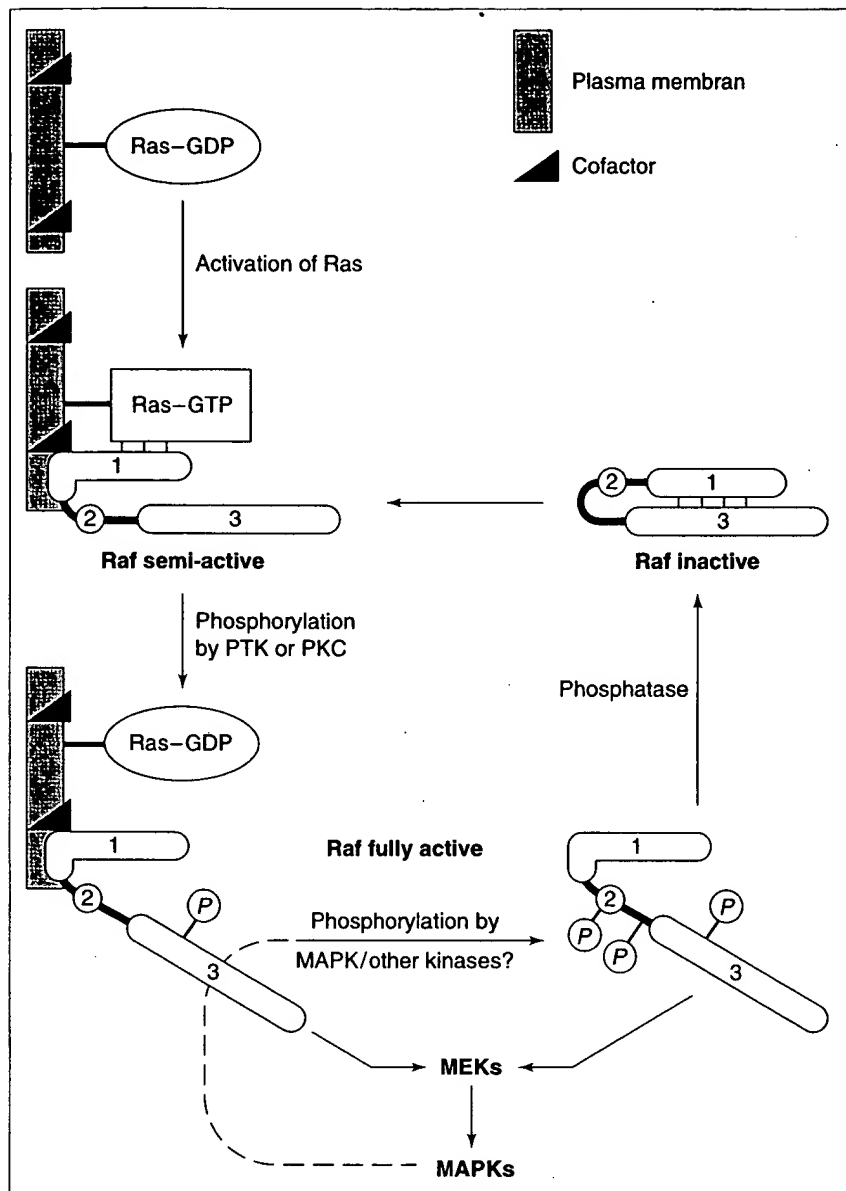


Figure 2

Model for Raf activation. Upon docking of c-Raf-1 via Ras-GTP at the membrane, a conformational change of the c-Raf-1 kinase occurs in that the hydrophobic face of the zinc-finger structure is stabilized by the lipid bilayer. We suggest that a so-far unknown cofactor is an essential part of that transition. This conformation of Raf frees the catalytic domain, resulting in a partial activation of the kinase. Maximal stimulation of Raf activity appears to require phosphorylation, catalysed by either serine/threonine or tyrosine kinases. Upon activation of the downstream effectors ERK1 and ERK2, feedback phosphorylation of Raf occurs, resulting in its dissociation from the membrane. Numbers refer to the conserved regions of the Raf molecule.

phosphorylated in unstimulated cells, suggesting that phosphorylation of this site is a co-translational event rather than part of c-Raf-1 activation. Mutation of Ser621 to alanine led to an inactive Raf-1 kinase that could not be activated by coexpression in insect cells with PKC- α or a combination of Ras and Lck (a member of the Src PTK family). This finding can be explained in two ways. First, activation resistance of the

mutant protein might be due to its lack of phosphorylation. This would introduce a new level of regulation, enabling the cell to switch off c-Raf-1 kinase by dephosphorylation of this site. Second, the Ser621Ala mutation may result in denaturation of the kinase. A similar situation apparently exists in the case of MEK1, where exchange of either of the two activating serine residues to alanine resulted in an enzyme that was

resistant to activation by Raf. By contrast, when one of these serines was replaced by threonine, which allows sequential dephosphorylation of the sites, it was seen that phosphorylation of either Ser218 or Ser222 was sufficient to activate MEK1 (Ref. 22). The simplest explanation for these findings is a denaturing effect of the alanine exchange.

The Ser259Ala mutant exhibited a twofold higher basal activity than wild-type c-Raf-1 and showed transforming activity in NIH3T3 cells. These findings can be explained by assuming a conformational change resulting in activation of c-Raf-1 kinase. Consistent with this hypothesis is the fact that duplication of the tetrapeptide 256-259 by linker-insertion mutagenesis was activating².

Exchange of Ser43 to alanine had no effect on c-Raf-1 activity. This site becomes phosphorylated *in vitro* and *in vivo* upon activation of cyclic-AMP-dependent protein kinase (PKA). While this phosphorylation does not affect the activity of previously activated Raf²¹, it is inhibitory to Ras-mediated activation²³. A possible mechanism lies in attenuation of Ras-Raf binding, perhaps by generation of increased binding activity between amino-terminal residues 1-50 and the RBD. If phosphorylation of Ser43 is the sole explanation for PKA inhibition of Ras-mediated Raf activation, it might be expected that A- and B-Raf would be resistant to the PKA-mediated blockade of activation, since these isozymes lack the Ser43 phosphorylation site.

There are two instances in which activating phosphorylation sites on Raf were identified: those used by PKC- α and those used by Src. PKC- α phosphorylates c-Raf-1 at Ser499 and presumably also at Ser259 in phorbol-ester-treated fibroblasts. Exchange of Ser499 for alanine had no effect on basic c-Raf-1 activity but strongly attenuated its activation when coexpressed with PKC- α in insect cells. This mutant, however, was not altered in its response to Ras-Lck¹⁷. Interestingly, another isozyme of the PKC family, the phospholipid-independent PKC- ϵ , might also play a role in Raf activation. Overexpression of the phosphatidylcholine-specific phospholipase C (PC-PLC) could bypass the block to proliferation caused by dominant-negative *ras* and lead to activation of Raf²⁴. Recently, Cai *et al.*²⁵ suggested that PKC- ϵ is activated by PC-PLC, but it remains to be demonstrated whether PKC- ϵ can phosphorylate and activate Raf.

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Analysis of Src-regulatory sites on Raf used coexpression of Src with c-Raf-1 and Ras in insect cells and led to the identification of two tyrosine phosphorylation sites, Tyr340 and Tyr341. Substitution of these residues with the negatively charged aspartic acid, presumably mimicking the phosphorylated status, increased the basal activity of c-Raf-1. Exchange of both Tyr340 and Tyr341 to phenylalanine resulted in an inactive protein that was resistant to activation by Ras-Lck²⁶ and, surprisingly, also to PKC- α (G. Heidecker and U. R. Rapp, unpublished). It would be interesting to replace these tyrosine residues with residues other than phenylalanine to test whether the PKC resistance was due to denaturation of the c-Raf-1 mutant. Interestingly, B-Raf contains two negatively charged residues in place of Tyr340/341, and initial experiments indicate that it shows elevated basal activity in the baculovirus expression system (U. R. Rapp, unpublished).

Tyrosine phosphorylation of c-Raf-1 was also observed *in vivo*. For example, stimulation of CTLL-2 T cells with interleukin-2 resulted in high-stoichiometry phosphorylation of c-Raf-1 on tyrosine. Incubation with phosphotyrosine-specific phosphatases, but not with serine/threonine-specific phosphatases, completely inactivated c-Raf-1 (Ref. 27). These findings, however, appear to be specific for the CTLL-2 cell line since they were not observed in human primary T cells.

In addition to stimulatory phosphorylations and others that interfere with Ras-dependent Raf activation, there is a third category of apparently neutral phosphorylation events. Raf phosphorylation by MAPKs is such a case, as there is good evidence for the ability of ERK1 and ERK2 to phosphorylate c-Raf-1 without activating the kinase²⁸. A possible function for this feedback phosphorylation may be the dissociation of activated Raf from the membrane, as illustrated in Fig. 2.

Raf-binding proteins

Given the direct binding between Raf and Ras, as well as between Raf and its substrate, MEK, it is reasonable to suspect that additional important elements in Raf signalling might be found by examining Raf-binding proteins. Traditional approaches, including co-immunoprecipitation and affinity chromatography, initially identified potential Raf activators such as RTKs or PTKs as

Raf-binding proteins. Raf binding was mitogen dependent in the case of RTKs and Src, or required activation of the T-cell-specific CD4 receptor in the case of Lck²⁰. The mitogen dependency of Raf binding may be explained by the creation of specific interaction sites by phosphorylation events subsequent to receptor stimulation. A clear-cut case for stimulation of specific protein binding by serine phosphorylation has recently been reported for the nuclear coactivator protein CREB-binding protein (CBP), which binds specifically to the PKA-phosphorylated form of the transcription factor CREB²⁹.

In contrast to mitogen-induced Raf association with RTKs and PTKs, in two cases, hypophosphorylated Raf was shown to associate with receptors before receptor stimulation. Raf bound to the γ - and δ -chains of CD3, a component of the T-cell-receptor-CD3 complex³⁰ from which it dissociated upon receptor stimulation. It was suggested that Raf is phosphorylated and activated by PTKs associated with the activated T-cell-receptor-CD3 complex. Binding of Raf to the unstimulated receptor might prime the kinase for rapid activation by receptor-regulated Raf kinase kinases. Considering the *a priori* proximity of Raf to the membrane, it would be interesting to know whether Ras is still required for its activation.

More recently, the yeast two-hybrid system was used to identify new Raf-binding proteins. Using Raf CR1 as bait, this system has yielded Raf inhibitors as well as potential coactivators. The small GTP-binding protein Rap1b (also known as K-rev-1), which is 95% identical to Rap1a, is one example of a Raf inhibitor discovered using this technique. These proteins are identical to Ras in their effector domains. Expression of Rap1b reverted the transformed phenotype of Ki-v-Ras-transformed NIH3T3 cells⁹. It is plausible that the underlying mechanism was the formation of an inactive Rap1b-Raf complex.

An example of a potential coactivator is provided by the members of the 14-3-3 protein family (Refs 31, 32; J. Avruch, pers. commun.). The 14-3-3 proteins are widely distributed and evolutionarily highly conserved. A brain-derived member of this family was shown to inhibit PKC. Taking into account the structural similarities between PKC and Raf, a regulatory role of membrane-associated forms of 14-3-3 proteins in Raf activation is conceivable. Membranes were

the source of a factor that enabled GTP-bound Ras, but not GDP-bound Ras, to mediate Raf activation in *in vitro* reconstruction experiments with subcellular fractions derived from bovine brain (T. Kamata and U. R. Rapp, unpublished). These experiments suggest that an intact lipid bilayer or a lipid activator is necessary for stimulation of Raf, since the membrane-derived coactivator was detergent labile.

Another Raf-associated protein, Bcl-2, neither affected Raf kinase activity nor served as a substrate, but synergized with Raf in the suppression of apoptosis. This interaction involved the CR3 domain of Raf but did not require a functional kinase domain. The basis of synergism may be targeting of the Raf kinase to substrates important for cell survival³³.

Downstream of Raf

At present, MEK1 and MEK2 are the only known physiological substrates of c-Raf-1. The Raf-specific phosphorylation sites have been determined for MEK1. Two serine residues at positions 218 and 222 become phosphorylated, and either is sufficient for activation²². Both sites are located between protein kinase subdomains VII and VIII. This is also the region where activating phosphorylations were observed in a number of other growth-regulatory protein kinases, including Src family PTKs, p34^{cdc2}, MAPK and PKA²². Activated forms of A- and B-Raf probably overlap in substrate specificity with c-Raf-1, as they could activate an epitope-tagged version of ERK1 upon coexpression in a human tumour cell line¹¹. Other kinases known to activate and phosphorylate MEK are Mos and MEK kinase (MEKK)^{9,34}. A remarkable feature of MEK phosphorylation by Raf, as well as MAPK phosphorylation by MEK, is the extremely high specificity of the reaction. As both kinases recognize the native protein rather than consensus substrate sequences, complex formation with the whole substrate protein must be an important guiding principle.

The MEK-binding domain on Raf was examined by use of the two-hybrid method, and shown to correspond to CR3 (Ref. 9). This was consistent with the fact that MEK phosphorylation and activation was first demonstrated for truncation-activated forms of Raf kinase⁹.

In contrast to Raf and MEK, ERK1 and ERK2 act on a variety of effector molecules, which were the focus of a

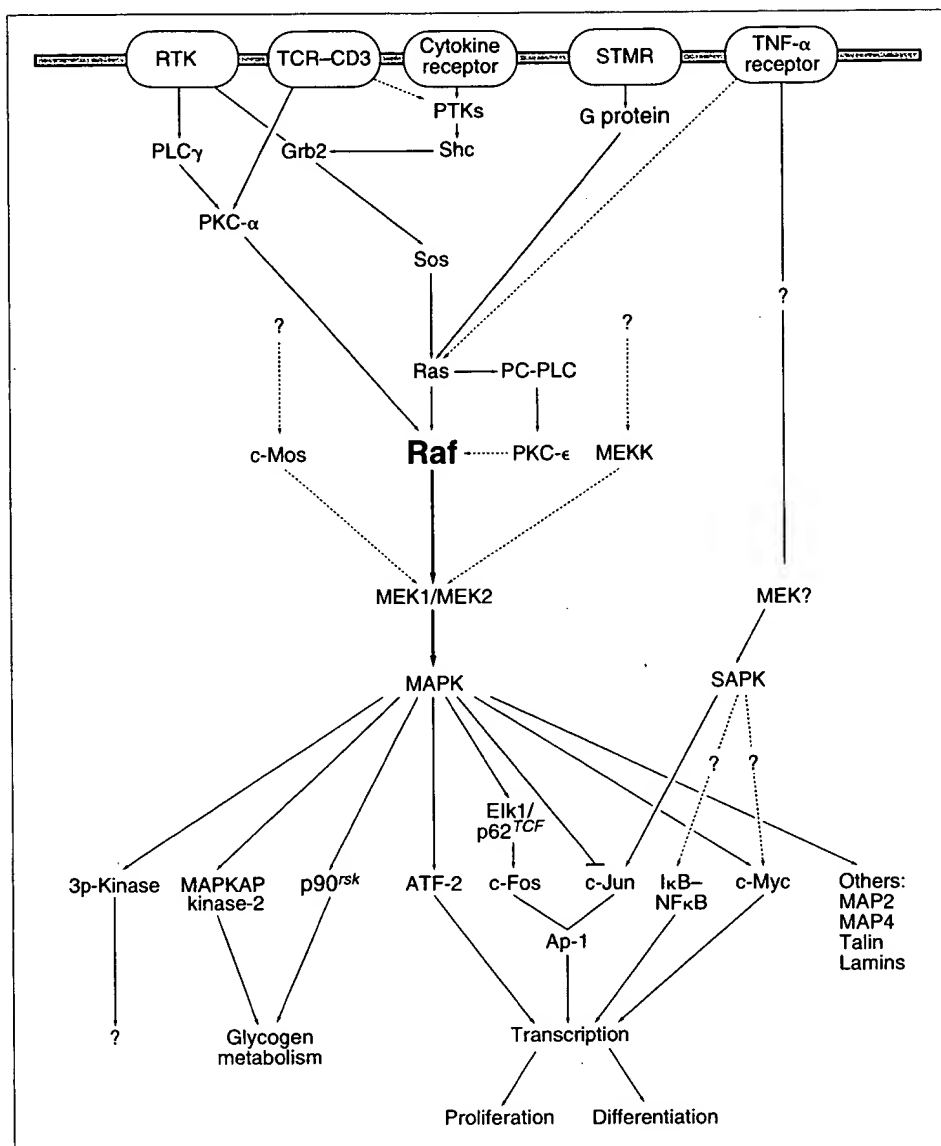


Figure 3

Raf-dependent signal transduction. For clarity, additional pathways, such as JAK/STAT-mediated signalling, are omitted, as are feedback phosphorylation reactions. Raf is activated upon stimulation of a variety of receptors. Together with MAP/ERK kinase (MEK) and mitogen-activated protein kinase (MAPK; also known as extracellular-receptor-activated protein kinase, ERK) it forms the highly conserved cytoplasmic kinase cascade. MAPK acts on numerous effector molecules, such as other serine/threonine kinases or transcription factors, which finally determine the cellular response. See text for details. The 3p-kinase is a novel protein kinase that is encoded by a gene on the short arm of chromosome 3, and is related to MAPK-activated protein kinase 2 (MAPKAP kinase-2; G. Sithanandam, pers. commun.). To indicate connections between different signal transduction pathways, tumour necrosis factor α (TNF- α) signalling, including stress-activated protein kinases (SAPKs)³⁹, is illustrated.

number of recent reviews^{34,35} (Fig. 3). Effectors include Ser/Thr kinases (pp90^{rsk}, MAPK-activated protein kinase-2 and 3p-kinase), transcription factors (Elk-1, c-Myc, c-Jun, NF- κ B and ATF-2) and structural proteins (talin, MAPs and lamins).

Are there alternative signal transduction pathways for induction of growth?

The essential nature of Raf for activation of the cytoplasmic kinase cascade

has been demonstrated for induced growth of NIH3T3 fibroblasts⁷, as well as for haemopoietic cell lines and primary bone-marrow cultures (K. Muszynski, pers. commun.). This conclusion was based on data obtained by three different approaches: expression of dominant-negative mutants, elimination of the protein using antisense RNA, and microinjection of inhibitory antibodies. Nevertheless, recent research has revealed the existence of

alternative MEK kinases, indicating that there is a Raf-independent mechanism for activation of MEK1 and MEK2. MEKK, a mammalian homologue of *Saccharomyces cerevisiae* STE11 kinase, was cloned recently and shown to activate MEK independently of Raf. Another candidate for a MEK kinase is Mos, which was shown to phosphorylate and activate MEK1 and MEK2 *in vitro*³⁴. Upstream activators are not known for either of these enzymes. We conclude that there are multiple inputs, at least at two levels of the cascade: Raf and MEK. What about signal output from these enzymes? We have to consider that there is branching at the level of c-Raf-1 or MEK1 and MEK2, since there is evidence for effects of Raf in cells in the absence of activation of ERK1 and ERK2. For example, while v-Raf-transformed NIH3T3 cells contain activated MAPK, no such activity was found in v-Raf-transformed Rat-1 fibroblasts. Similarly, interleukin-2 stimulates Raf kinase, but not MAPK activity³⁵. Finally, v-Raf abrogates the requirement for colony-stimulating factor 1 of macrophages without activating ERK1 or ERK2 (Ref. 36), reminiscent of the finding with the v-Raf-transformed Rat-1 fibroblasts.

There is also evidence for multiple growth-regulatory outputs at the level of Ras. A physiological switch from growth factor-activated Ras-Raf signalling to the use of an alternative Ras effector pathway can be induced in rat thyroid cells upon treatment with thyroid-stimulating hormone (TSH). TSH activates PKA, as well as Ras, via heterotrimeric G proteins, and is sufficient to induce proliferation in the absence of growth factors. The switch that TSH induces in the presence of growth factor presumably involves phosphorylation of Raf by PKA, resulting in a lowered Ras-binding activity²³. As a result, a different effector of Ras with the ability to cooperate with PKA signalling comes into play (N. Al-Alawi, unpublished). This switch induction is an illustration of the

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flexibility built into the wiring system for mitogenic signalling. Elements of flexibility include the existence of multiple Ras effectors, differences in their level of expression, their binding affinity for Ras and regulatory interactions between signalling pathways. Such crosstalk provides a mechanism for the coordinated recruitment of cooperative signal transducers. PI 3-kinase is a possible candidate for an alternative Ras effector in WRT rat thyroid cells, since its binding to Ras has been demonstrated recently in other cell systems¹⁴.

Cytokine receptors and some RTKs initiate an additional signal transduction pathway consisting of members of the so-called Janus kinase (JAK) family and latent transcription factors in the cytosol (STATs)³⁷. The relative contribution of the JAK pathway versus the cytoplasmic kinase cascade to growth induction by growth factors remains to be elucidated. Current indications are that both pathways are required.

Cellular responses to Raf kinase

A physiological role of Raf was demonstrated in the development of invertebrates. In *Drosophila*, mutations in D-Raf interrupted signalling through the torso and sevenless RTKs, resulting in alteration of terminal structures and the R7 photoreceptors, respectively³. The Raf homologue in *C. elegans*, Ce-Raf, is required for vulval differentiation⁴. In mammalian cells, experiments focused initially on transformation and proliferation in NIH3T3 fibroblasts and several haemopoietic cell lineages. Recent work has shown that the Raf kinase also plays a decisive role in other cellular responses, such as differentiation and survival. For example, Raf was shown to mediate insulin-induced differentiation of 3T3L1 cells to adipocytes³⁸ as well as nerve growth factor-dependent neurite outgrowth in pheochromocytoma (PC12) cells¹⁹. Induction of differentiation by *v-raf* was also observed in mice, where stimulation of erythroid cell differentiation was most dramatic⁷. Furthermore, an involvement of Raf in survival was shown in the myeloid cell line 32Dcl.3. These cells require interleukin-3 (IL-3) for proliferation, and undergo apoptosis upon growth factor abrogation. In the absence of IL-3, expression of *v-raf* alone suppressed, whereas *v-myc* expression accelerated, programmed cell death. Concomitant expression of both resulted in growth of the cells in the absence of IL-3 (Ref. 7). We

conclude that a balance of individually antagonistic signalling pathways activated by the same growth factor receptors is required for orderly progression through the cell cycle. Although there is evidence for the existence of multiple independent regulators and substrates at each tier of the cascade, these options are probably not exercised simultaneously. This is suggested by genetic epistasis experiments in *Drosophila*³ and *C. elegans*⁴, which did not provide evidence for signal loss when an upstream element of the cascade, such as the receptor, Ras or Raf, was inactivated and replaced by a downstream effector. As illustrated by the PKA-induced Ras-effector switch, it may be more likely that the use of other substrates by Raf and MEK is regulated in an all-or-none fashion.

It will be interesting to test whether MAPK function can similarly be replaced by downstream substrates. The hour-glass model illustrated in Fig. 3 predicts that no individual substrate would be functionally equivalent to MAPK.

Future perspectives

Raf kinases have been established as critical gatekeepers in growth factor signal transduction and oncogenic transformation. Major questions remain regarding activation, novel substrates and isozyme-specific functions of Raf kinases. An additional topic for examination is the possibility of switches that allow for crosstalk between different signalling pathways, as shown for the cAMP-mediated inhibition of the cytoplasmic kinase cascade in WRT thyroid cells. Another switch mechanism at the level of Ras may be provided by the binding of other small GTP-binding proteins to Raf, as demonstrated in the case of Ras and Rap1a binding to c-Raf-1. The range of this flexibility has to be probed and the cellular processes identified that are regulated by growth factors in this way. We have profound knowledge of short-term effects mediated by Raf resulting in transcriptional regulation. However, there are many gaps in our understanding of the nature of the processes leading to long-term changes, such as cell-cycle progression, suppression of apoptosis and induction of differentiation.

Finally, in the light of recent data on the occurrence of point-mutated *c-raf-1* in mouse lung tumours, efforts should be made to determine the status of *raf* genes in human cancer. In the

mouse model, consistent amino acid exchanges were observed that are apparently all located at the surface of the substrate pocket (S. M. Storm and U. R. Rapp, unpublished). If a similar clustering of mutations is observed in human tumours it may be possible to develop inhibitors that can distinguish between normal and oncogenic Raf kinase, a dream that is as old as the discovery of the first oncogene in human tumours.

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Owing to TIBS' policy of short reference lists, the number of references cited in this article has been limited. Some work has therefore unfortunately been left unacknowledged.

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THE IMPORTANCE OF protein tyrosine kinases (PTKs) and tyrosine phosphorylation in controlling such fundamental cellular processes as growth and differentiation is now well established. However, it is important to realize that, in the cell, tyrosine phosphorylation is a reversible, dynamic process. Thus, the net level of phosphate in a target substrate reflects not only the activity of the PTKs that catalyze phosphorylation, but also the protein tyrosine phosphatases (PTPs) that are responsible for dephosphorylation of tyrosyl residues. It is now clear that, like the PTKs, the PTPs are a large and structurally diverse family of receptor-like and cytoplasmic enzymes. They have been identified in sources as diverse as mammals, *Xenopus*, *Drosophila*, *Caenorhabditis elegans*, *Dictyostelium*, yeast, prokaryotes and viruses. Their structures suggest important functions including control of cell-cell adhesion, growth factor signal transduction, and the cell cycle, and dysfunctional PTPs have been implicated in disease states. The characterization of the PTP family now represents a major research effort in many laboratories (for detailed reviews, see Ref. 1).

In this review we have tried to illustrate examples of the integration of PTP and PTK activity in the control of cell function. The PTPs and PTKs do not exert their effects in isolation, but rather coordinate their effects in controlling flux through tyrosine-phosphorylation-dependent signaling pathways.

PTPs antagonize the action of PTKs

Since deregulated PTKs have been found to be encoded by oncogenes, it has been assumed that PTPs may function as products of anti-oncogenes. Indeed, inactivating mutations in genes encoding PTPs may contribute to the aberrant tyrosine phosphorylation associated with certain neoplastic

The levels of tyrosine phosphorylation required for cell growth and differentiation are achieved through the coordinated action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Depending upon the cellular context, these two types of enzymes may either antagonize or cooperate with each other during the signal transmission process. An imbalance between these enzymes may impair normal cell growth, leading to cellular transformation. Both PTKs and PTPs have evolved to a level of structural diversity that allows them to regulate many cellular processes. This review will focus on several specific examples that highlight the interplay between PTPs and PTKs in cell signaling.

conditions. Considerable progress has been made in the determination of the chromosomal localization of genes encoding PTPs, with the aim of correlating map positions with sites of abnormality in human disease states. New and tantalizing results are being reported frequently. Most notable is the gene for PTP γ on chromosome 3p21, a segment frequently altered in renal and small-cell lung carcinomas. It now appears that mutations may occur in the extracellular segment of PTP γ and thus may create a receptor-like PTP that can no longer respond to its external signals².

Several studies have reported the inhibitory effects of PTPs on cellular transformation in tissue culture systems. Expression of PTP1B has been shown to block transformation mediated by Neu³ and partially revert transformation by Src⁴, both of which are oncogenic PTKs. Expression of a closely related phosphatase, TCPTP, has been shown to reverse partially the morphological phenotype of cells transformed by Fms, a deregulated colony-stimulating factor receptor⁵. At least in

the case of TCPTP, the reversion of transformation has been found to be accompanied by the disappearance of a subset of tyrosyl-phosphorylated proteins, indicating a degree of substrate specificity for the PTP *in vivo*. The targets of PTPs may be PTKs themselves or the substrates of PTKs (Fig. 1). While more comprehensive studies are needed to define the substrates critical for the transformed phenotype, the results to date convey the idea that PTPs may be powerful antagonists of the actions of normal cellular and oncogenic PTKs.

More recently, a growth-suppressor function for a cytoplasmic PTP has been characterized, illustrating the power of a genetic approach to the elucidation of PTP function. HCP (also known as PTP1C, SH-PTP1 or SHP; for reviews, see Refs 6, 7) is a hemopoietic-cell PTP characterized by two Src homology 2 (SH2) domains in its amino-terminal segment. SH2 domains have been shown to bind phosphotyrosyl residues in defined primary sequences⁸. Mutations in the gene encoding HCP that result in aberrant splicing of its

The coordinated action of protein tyrosine phosphatases and kinases in cell signaling

Hong Sun and Nicholas K. Tonks

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<u>Set Name</u> side by side	<u>Query</u>	<u>Hit Count</u>	<u>Set Name</u> result set
<i>DB=USPT; PLUR=YES; OP=ADJ</i>			
<u>L9</u>	inhibit raf kinase	1	<u>L9</u>
<u>L8</u>	raf inhibitor\$1	2	<u>L8</u>
<u>L7</u>	raf inhibitor@1	0	<u>L7</u>
<u>L6</u>	inhibitor\$1 raf-1	3	<u>L6</u>
<i>DB=USPT; THES=ASSIGNEE; PLUR=YES; OP=ADJ</i>			
<u>L5</u>	L4 and inhibit\$3	103	<u>L5</u>
<u>L4</u>	L3 and (mapk or erk)	103	<u>L4</u>
<u>L3</u>	L1 and kinase\$1	223	<u>L3</u>
<u>L2</u>	L1 and rkip	0	<u>L2</u>
<u>L1</u>	raf-1	235	<u>L1</u>

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L4	L1 and kinase\$1	223	L4
L3	L1 and kinase@1	0	L3
L2	L1 and rkip	0	L2
L1	raf-1	235	L1

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L7: Entry 1 of 5

File: DWPI

Jun 13, 2000

DERWENT-ACC-NO: 2000-411281

DERWENT-WEEK: 200221

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TITLE: Novel mitogen extracellular signal-regulated kinase kinase (MEKK) protein useful for treating cancer, inflammation, autoimmune diseases, neurological disorders and hormone related disease in animals

INVENTOR: JOHNSON, G L

PRIORITY-DATA: 1995US-0440421 (May 12, 1995), 1993US-0049254 (April 15, 1993), 1994WO-US04178 (April 15, 1994), 1994US-0323460 (October 14, 1994), 1994WO-US11690 (October 14, 1994), 1995US-0354516 (February 21, 1995), 1995US-0461145 (June 5, 1995)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 6074861 A	June 13, 2000		092	C12N009/12

INT-CL (IPC): C07 H 21/04; C07 K 1/00; C12 N 9/12

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	Claims	RnM	Draw Desc	Image
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☐ 2. Document ID: US 5981265 A

L7: Entry 2 of 5

File: DWPI

Nov 9, 1999

DERWENT-ACC-NO: 1999-633328

DERWENT-WEEK: 200221

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TITLE: Regulating mitogen extracellular signal-regulated kinase kinase protein activity, useful for the treatment of cancer, neurological diseases and autoimmune diseases

INVENTOR: JOHNSON, G L

PRIORITY-DATA: 1995US-0440421 (May 12, 1995), 1993US-0049254 (April 15, 1993), 1994WO-US04178 (April 15, 1994), 1994US-0323460 (October 14, 1994), 1994WO-US11690 (October 14, 1994), 1994US-0345516 (November 28, 1994), 1995US-0461146 (June 5, 1995)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 5981265 A	November 9, 1999		094	C12N001/15

INT-CL (IPC): C12 N 1/15

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments	Claims	RnM	Draw Desc	Image
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☐ 3. Document ID: US 5854043 A

L7: Entry 3 of 5

File: DWPI

Dec 29, 1998

DERWENT-ACC-NO: 1999-094912

DERWENT-WEEK: 200221

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TITLE: Mitogen activated protein kinase kinases and their fragments - used for regulating signalling from growth factor receptors, e.g. to modulate apoptosis for treatment of cancer, autoimmune disease and inflammation

INVENTOR: JOHNSON, G L

PRIORITY-DATA: 1994US-0323460 (October 14, 1994), 1993US-0049254 (April 15, 1993), 1994WO-US04178 (April 15, 1994)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 5854043 A	December 29, 1998		096	C07K014/435

INT-CL (IPC): C07 K 14/435; C12 N 9/12

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequences	Attachments
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RMIC	Draw Desc	Image
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☐ 4. Document ID: WO 9528421 A1 AU 9480177 A EP 755406 A1 JP 09511906 W KR 97702294 A AU 703070 B CA 2186526 C

L7: Entry 4 of 5

File: DWPI

Oct 26, 1995

DERWENT-ACC-NO: 1995-373762

DERWENT-WEEK: 200035

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TITLE: New signal-regulated kinase proteins and nucleic acids - used for regulating cell responsiveness for treating e.g. tumours, auto-immune disease, inflammation or neuronal disorders.

INVENTOR: JOHNSON, G L

PRIORITY-DATA: 1994WO-US04178 (April 15, 1994)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
WO 9528421 A1	October 26, 1995	E	147	C07K014/00
AU 9480177 A	November 10, 1995		000	C07K014/00
EP 755406 A1	January 29, 1997	E	000	C07K014/00
JP 09511906 W	December 2, 1997		126	C12N015/09
KR 97702294 A	May 13, 1997		000	C07K014/00
AU 703070 B	March 11, 1999		000	C07K014/00
CA 2186526 C	December 14, 1999	E	000	C12N015/54

INT-CL (IPC): A61 K 31/70; A61 K 35/76; A61 K 38/00; A61 K 38/17; A61 K 48/00; C07 H 21/00; C07 H 21/04; C07 K 14/00; C12 N 9/12; C12 N 15/00; C12 N 15/09; C12 N 15/54; C12 Q 1/00; C12 Q 1/48; C12 Q 1/68; G01 N 33/573; C12 N 9/12; C12 R 1/19

[Full](#) [Title](#) [Citation](#) [Front](#) [Review](#) [Classification](#) [Date](#) [Reference](#) [Sequences](#) [Attachments](#)[KIMC](#) [Draw Desc](#) [Image](#)

☐ 5. Document ID: WO 9424159 A1 AU 9466663 A US 5405941 A FI 9504894 A NO 9504094 A EP 694044 A1 JP 08509128 W NZ 266067 A AU 697340 B

L7: Entry 5 of 5

File: DWPI

Oct 27, 1994

DERWENT-ACC-NO: 1994-357747

DERWENT-WEEK: 200221

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TITLE: New MEK kinase protein and related antibodies and nucleic acid - regulator of mitogen activated protein kinase, useful therapeutically to inhibit cell atrophy, to screen for oncogenes etc.

INVENTOR: JOHNSON, G L

PRIORITY-DATA: 1993US-0049254 (April 15, 1993)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
WO 9424159 A1	October 27, 1994	E	084	C07K013/00
AU 9466663 A	November 8, 1994		000	C07K013/00
US 5405941 A	April 11, 1995		029	C07K013/00
FI 9504894 A	October 13, 1995		000	C07K000/00
NO 9504094 A	December 13, 1995		000	C07K000/00
EP 694044 A1	January 31, 1996	E	000	C07K013/00
JP 08509128 W	October 1, 1996		072	C12N015/09
NZ 266067 A	September 22, 1997		000	C12N009/12
AU 697340 B	October 1, 1998		000	C12N009/12

INT-CL (IPC): [A61 K 37/02](#); [A61 K 37/52](#); [A61 K 38/45](#); [C07 H 21/04](#); [C07 K 0/00](#); [C07 K 13/00](#); [C07 K 15/28](#); [C07 K 16/40](#); [C12 N 9/12](#); [C12 N 15/09](#); [C12 N 15/12](#); [C12 P 21/08](#); [C12 Q 1/02](#); [G01 N 33/53](#); [G01 N 33/573](#); [C12 N 9/12](#); [C12 R 1/91](#)[Full](#) [Title](#) [Citation](#) [Front](#) [Review](#) [Classification](#) [Date](#) [Reference](#) [Sequences](#) [Attachments](#)[KIMC](#) [Draw Desc](#) [Clip Img](#) [Image](#)[Generate Collection](#)[Print](#)

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